0273-2289/01/90/0211/$12.50

Purification of α-Amylases Using Magnetic Alginate Beads

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Received April 1, 2000; Revised August 1, 2000; Accepted August 1, 2000

Abstract

Magnetic alginate beads were used to purify α-amylases from porcine pancreas, starchzyme, BAN 240L (a commercial purification from *Bacillus subtilis*), and wheat germ. The beads bound a significant level of α-amylase activity from porcine pancreas, BAN 240L, and wheat germ. In each case, the enzyme activity could be eluted by using 1.0 *M* maltose, a known competitive inhibitor of α-amylase. In the case of BAN 240L, 3.6-fold purification with 72% recovery of activity was observed. In the case of wheat germ enzyme, starting from the crude extract, 48-fold purification with 70% activity recovery was observed. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis also indicated considerable purification in the latter case.

Index Entries: Affinity separation; α-amylase; magnetic alginate beads; macroaffinity ligands; wheat amylase.

Introduction

Magnetic methods of bioseparation have several advantages in comparison with other techniques for protein/enzyme separation *(1–3)*. One major advantage is that one can directly work with viscous solutions and crude suspensions. Although biomagnetic separation is extensively used for analytical purposes *(4,5)*, quite a few successful applications for separation of protein/enzyme have also been reported *(1,2)*.

Recently, we have reported that alginates can act as macroaffinity ligands for α-amylases *(6)*. α-Amylases are industrially important enzymes and find applications in starch modification and degradation *(7)*. Alginates are polysaccharides of marine origin. These polysaccharides are copolymers of mannuronic and guluronic acid. Being nontoxic, alginates are widely used in food industries *(8)*.

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In the present work, we describe the use of magnetic alginate beads for separation of α-amylase activity from porcine pancreas using two commercial preparations from microbial sources and wheat germ. We also describe the considerable purification of the enzyme directly from crude wheat germ extract.

Materials and Methods

Sodium alginate (cat. no. A-2158; with high content of mannuronic acid residues), wheat germ powder, and α-amylase (porcine pancreas) were purchased from Sigma (St. Louis, MO). Protanal LF 10/60 (from brown seaweed, having a high guluronic acid content of 65–75%) was a gift from Prof. Bo Mattiasson, Sweden. Starchzyme was purchased from Jaysons Agritech Pvt., Mysore, India, and was an industrial preparation of the enzyme from *Aspergillus oryzae*. BAN 240L (*Bacillus subtilis*) was generously supplied by NOVO Nordisk A/S (Bangalore, India). All other chemicals were of analytical grade.

Estimation of Enzyme Activity

α-Amylase activity was measured by using starch as the substrate *(9)*. One enzyme unit (U) was defined as the amount of enzyme that liberated 1 µmol of reducing sugar (calculated as maltose) per minute at 25°C. In the case of pig pancreas, the assay was carried out in 20 m*M* Tris-HCl buffer, pH 6.9, whereas in all other cases, 50 m*M* acetate buffer, pH 5.6, was used for the enzyme assay (NOVO Nordisk A/S [1990] product sheet on BAN).

Estimation of Protein

Protein was estimated by the dye-binding method *(10)* using bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the samples using 12% gel was performed according to Hames *(11)* on a Genei gel electrophoresis unit (Bangalore Genei Pvt., Bangalore, India) using standard molecular weight markers (Sigma).

Preparation of Alginate Beads

Sodium alginate (high mannuronic acid content) beads were prepared by dropping 50 mL of 2% alginate solution through a syringe into 100 mL of 0.1 *M* $CaCl_2$ solution. The beads obtained were incubated for 1 h in the same $CaCl_2$ solution *(12)*. The beads were finally stored in a 6 m*M* $CaCl_2$ solution at 4°C. The size of the beads made has been described to be 0.8 ± 0.03 mm by Burns et al. *(13)*. Our estimate by scanning electron microscopy (SEM) falls within this range.

Preparation of Magnetite

To 1.6 L of distilled water 12.8 g of $FeCl_2 \cdot 4H_2O$ and 34.56 g of $FeCl_3 \cdot 6H_2O$ were added and heated to 70°C *(13)*. Thirty-two grams of NaOH dissolved in 320 mL of distilled water was added to this heated solution. A black precipitate was immediately formed and settled out after standing at room temperature for 1 h. Later, part of the supernatant was aspirated, and the remaining magnetite suspension was extensively washed with several volumes of water.

Preparation of Magnetic Alginate Beads

Magnetic alginate beads *(13)* were prepared from two different types of alginates—sodium alginate (Sigma) and Protanal LF 10/60—keeping the magnetite concentration constant in both cases. Beads were formed by dropping a 50-mL mixture of 2% alginate solution and 2.2% magnetite (Fe_3O_4) through a syringe into a 100-mL 0.1 *M* $CaCl_2$ solution. The beads obtained were incubated for 1 h with the same concentration of $CaCl_2$ solution that was used for the bead formation. The beads were stored in a 6 m*M* $CaCl_2$ solution at 4°C. Alginate magnetite beads were the same size as the nonmagnetic alginate beads described above.

Extraction of Wheat Germ Amylase from Wheat Germ Powder

The amylase preparation *(6)* was obtained by stirring 10 g of wheat germ powder with 10 mL of chilled acetone in cold. After 2 h, 20 mL of 50 m*M* acetate buffer, pH 5.6, was added. The mixture was stirred in cold for 4 h. Then the extract was centrifuged at 12,000*g* for 15 min at 4°C. The clear supernatant obtained was used as crude wheat germ amylase preparation.

Binding of Amylase from Different Sources with Magnetic Alginate Beads

One milliliter of α-amylase from BAN 240L (8.80 U), starchzyme (8.80 U), and wheat germ (9.25 U) in 50 m*M* acetate buffer (pH 5.6) containing 6 m*M* $CaCl_2$ and 1 mL of α-amylase from porcine pancreas (8.80 U) in 20 m*M* Tris-HCl buffer (pH 6.9) containing 6 m*M* $CaCl_2$ were added separately to magnetic alginate beads preequlibrated with the same buffer as the corresponding enzyme, respectively. The volume of the beads was 3 mL (the volume occupied after the excess buffer has been drained off).

Similar sets of experiment were performed using magnetic alginate beads prepared from two different kinds of alginates (differing in monomer composition). In each case, after incubation for 1 h at 25°C, the supernatant was separated from the beads. The beads were washed with 1 mL of 50 m*M* acetate buffer (pH 5.6) containing 6 m*M* $CaCl_2$ or Tris-HCl buffer (in the case of porcine amylase) until no enzyme activity was detected in the washings. The enzyme activities were determined in the supernatants and

Table 1
Binding of Amylases to Alginate Magnetite Beads Made from Alginate of Different Compositions[a]

Type of alginate	Activity bound (%)			
	Porcine pancreas	Starchzyme	BAN 240L	Wheat germ
Sodium alginate (high mannuronic acid composition)	73	37	90	85
Protonal LF10/60 (high guluronic acid composition)	72	35	90	84

[a]The conditions for binding of the enzyme to alginate magnetite are as described in Materials and Methods.

washings. The difference between the enzyme activity of the supernatant (and washings) and the activity before the addition of magnetic alginate beads represented the amount of the enzyme activity bound.

Results and Discussion

α-Amylase from different sources (porcine pancreas, starchzyme, BAN 240L, wheat germ) was bound to magnetic alginate beads prepared from two different commercially available alginate preparations. The four amylase preparations of varying purity were used. Three of them, porcine pancreas, BAN 240L, and wheat germ, showed a significant level of binding to the magnetic alginate beads (Table 1). Alginate consists of residues of uronic acids of gulose and mannose that are stereoisomers of glucose (the monomer for starch that is the natural substrate for amylase) *(8)*. As discussed elsewhere *(6)*, it is likely that binding of α-amylase to alginate is via bioaffinity interactions.

In earlier work it has been found that the different kinds of α-amylases bind to a similar extent to the alginates made up of high mannuronic acid or high guluronic acid. In the present study (Table 1), both types of alginate also show similar binding trends. Alginate is obtained from the weeds of marine origin and the sequence of sugars depends on the particular weed *(8)*. Thus, it is reassuring that the binding of α-amylase is independent of the composition of the alginate. This implies that preparing these magnetite alginate beads from any commercial alginate can serve as an affinity material for binding.

Figures 1 and 2 show the surface structure of the alginate and magnetic alginate beads revealed by SEM. At X4500 magnification, one can see that the surface of the magnetic alginate bead is less structured. This effect on the morphology of the beads on incorporation of magnetite has not been

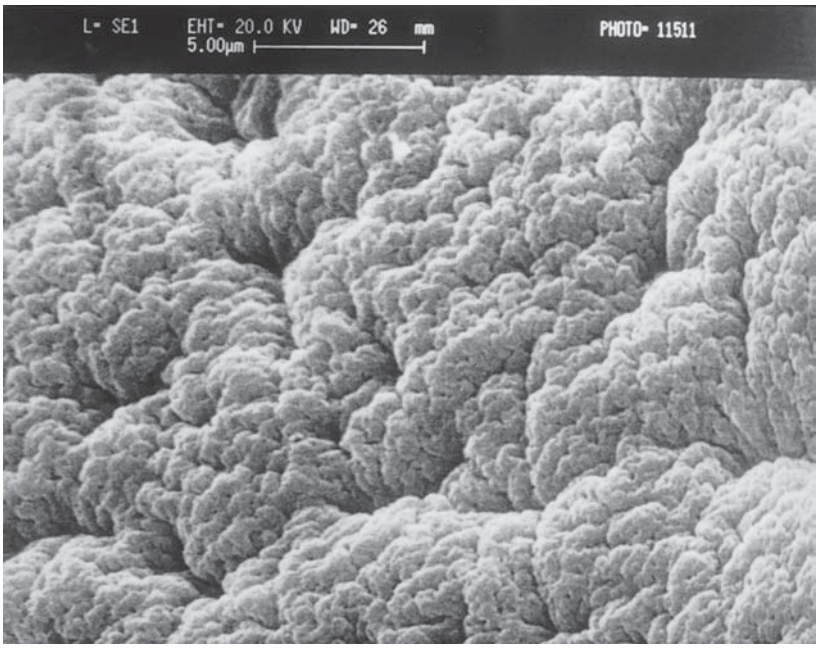

Fig. 1. Scanning electron micrograph of alginate bead taken at magnification X4500. Alginate beads were prepared as described in Materials and Methods.

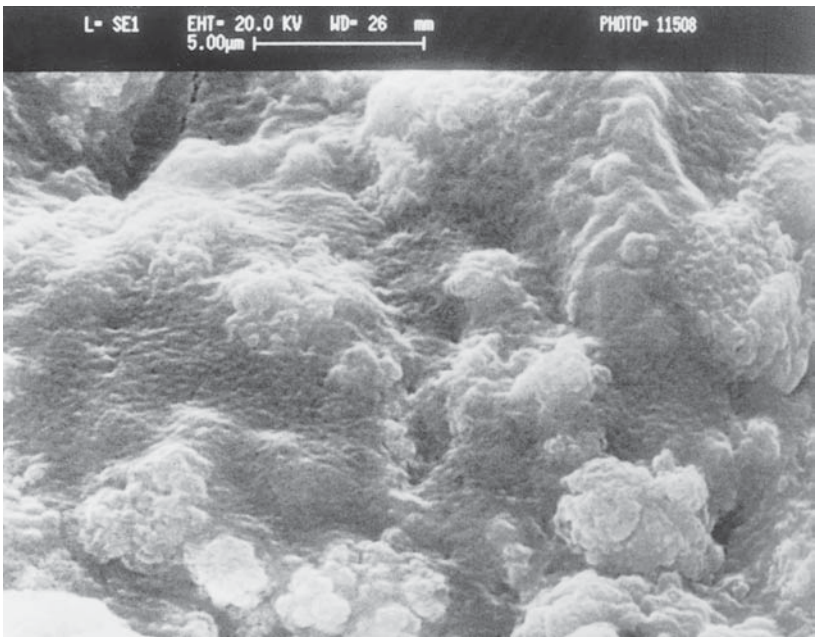

Fig. 2. Scanning electron micrograph of magnetic alginate bead taken at magnification X4500. Magnetic alginate beads were prepared as described in Materials and Methods.

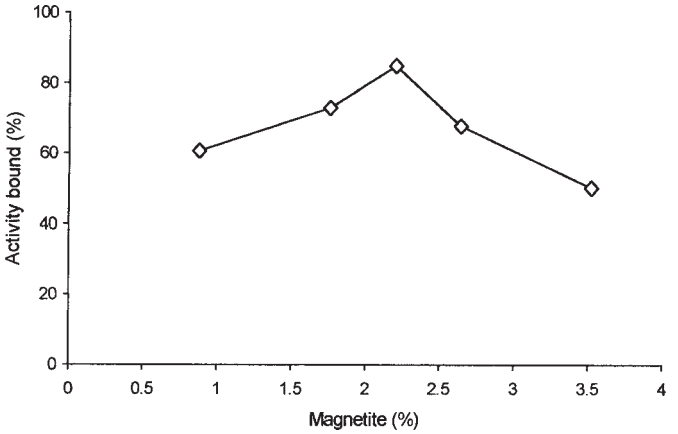

Fig. 3. Effect of magnetite concentrations on the binding of wheat germ α-amylase. Different concentrations of magnetite (0.88, 1.76, 2.20, 2.64, and 3.52%) were taken along a 2% alginate concentration in all cases. The amylase activity was determined using starch as substrate and the effect of different concentrations of magnetite present while making beads.

reported before. These micrographs indicate the spatially uneven distribution of magnetite in the beads.

As amylase preparation from porcine pancreas contained a rather low amount of protein, further purification work was restricted to BAN 240L and wheat germ amylase. The effect of varying the amount of magnetite along with constant alginate concentration was studied. The maximum bound activity was found when the ratio of concentration (% w/v) of magnetite to alginate was 1:1 (Fig. 3). Figure 4 shows the effect of enzyme load on the percentage of activity bound in the cases of BAN 240L (Fig. 4A) and wheat germ amylase (Fig. 4B).

Elution of the bound activity was tried with different eluting agents for BAN 240L and wheat germ. Tables 2 and 3 show that in both cases maltose specifically eluted the enzyme activity off the magnetic alginate beads. Maltose is known to be an inhibitor for α-amylase and has been used to elute this enzyme in other affinity protocols as well *(14)*. This further confirms that the binding of α-amylase to alginate is an example of affinity binding.

Table 4 shows the purification of BAN 240L. Because BAN 240L is a partially purified commercial preparation, fold purification is understandably not very high. However, final high specific activity of this purified preparation shows the considerable purification achievable with this approach.

As mentioned in the Introduction, one advantage of using magnetic methods is that we can work directly with crude suspensions even if these are viscous in nature. The crude enzyme extract from wheat germ thus constitutes a good test case for evaluating the usefulness of this approach. Table 5 summarizes the purification data concerning wheat germ enzyme.

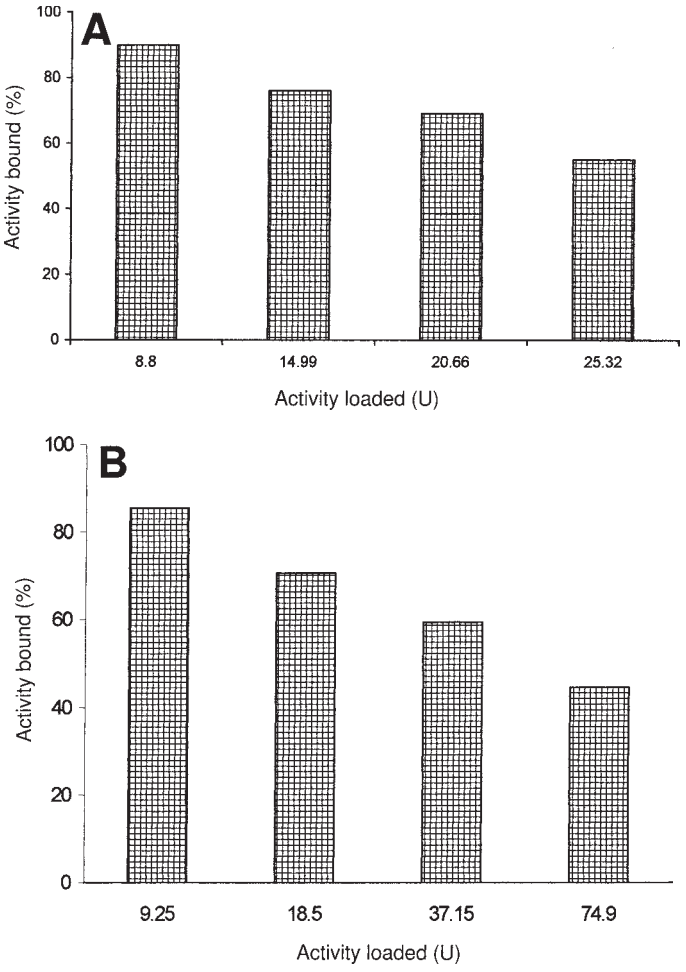

Fig. 4. Binding capacity of magnetic alginate beads for **(A)** BAN240L and **(B)** wheat germ amylase. BAN240L (8.80–25.32 U) and wheat germ amylase in 1 mL of 50 m*M* acetate buffer containing 6 m*M* $CaCl_2$, pH 5.6, were incubated with 3 mL of settled magnetic alginate beads for 1 h at 25°C. After 1 h of incubation, the beads were washed with 1 mL of buffer until no enzyme activity could be detected in the washings. The bound activity was calculated by subtracting the total activity present in the supernatant and washings from the initially added activity, which was taken to be 100%.

About 48-fold purification with 70% recovery of the enzyme activity shows what is possible in a single step using a magnetic affinity macroligand.

Figure 5 shows the SDS-PAGE pattern of the crude wheat germ amylase and the purified preparation. The present band corresponds to a molecular weight of 18 kDa, which agrees well with the value reported earlier for the enzyme *(15)*.

It may be interesting to compare the results obtained here with those reported earlier with other approaches. Simple alginate beads in the batch mode have given 45-fold purification with 70% activity recovery *(6)*. When

Table 2
Elution of Amylase BAN 240L
with Various Eluents[a]

Eluent	Eluted activity (%)
0.5 *M* maltose; 25°C (2 h)	22
0.5 *M* maltose; 4°C (4 h)	24
1.0 *M* maltose; 4°C (4 h)	72
1.0 *M* mannose; 4°C (4 h)	0
1.0 *M* glucose; 4°C (4 h)	37
1.0 *M* galactose; 4°C (4 h)	15
1.0 *M* sucrose; 4°C (4 h)	0
0.1 *M* borate; 4°C (4 h)	14
1.0 *M* NaCl; 25°C (1 h)	0

[a]The BAN 240L amylase was bound to the alginate magnetite beads as described in Materials and Methods. Enzyme (8.80 U) was added to the beads and quantitative binding of the enzyme activity was observed. Bound enzyme activity was recovered by incubating alginate magnetite beads (at temperatures indicated in each case) with 1.0 mL of sugar/borate/salt solution in 50 m*M* acetate buffer, pH 5.6. Extensive dialysis of the eluates was done against sodium acetate buffer before measuring the activity.

Table 3
Recovery of Wheat Germ Amylase[a]

Eluent	Eluted activity (%)
0.5 *M* maltose; 25°C (1 h)	25
0.5 *M* maltose; 4°C (1 h)	29
0.5 *M* maltose; 4°C (4 h)	40
1.0 *M* maltose; 4°C (4 h)	70
1.0 *M* mannose; 4°C (4 h)	0
1.0 *M* galactose; 4°C (4 h)	12
1.0 *M* sucrose; 4°C (4 h)	0
0.1 *M* borate; 4°C (4 h)	22
1.0 *M* NaCl; 25°C (1 h)	0
1.0 *M* glucose; 4°C (4 h)	30

[a]Wheat germ amylase (9.2 U) was bound to the alginate magnetite beads as described in Materials and Methods. Of the total activity, 85% was bound. Bound activity was recovered by incubating alginate magnetite beads (at the temperature indicated in each case) with 1.0 mL of sugar/borate/salt solution in 50 m*M* acetate buffer containing 6 m*M* $CaCl_2$, pH 5.6. Extensive dialysis of the eluates was done against sodium acetate buffer before measuring the activity.

Table 4
Purification of BAN 240L Amylase on Magnetite Alginate Beads[a]

Step	Activity (U)	Protein (μg)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude	8.8	10	880	100	1.0
Supernatant	0.9	—	—	—	—
Elution	6.4	2	3210	72.7	3.6

[a]All the experiments were carried out in triplicate and the difference in triplicates was less than ±5%. The binding of BAN 240L amylase activity to magnetic alginate beads was done as described in Materials and Methods. The bound activity was eluted by incubating the beads with 1 mL of 50 m*M* acetate buffer, pH 5.6, containing 1 *M* maltose at 4°C for 4 h. The activity was determined after extensive dialysis of maltose.

Table 5
Purification Table of Wheat Germ α-Amylase on Magnetic Alginate Beads[a]

Step	Activity (U)	Protein (μg)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude	9.2	610	15	100	1
Supernatant	1.3	80	—	—	—
Eluate	6.5	9	720	70	48

[a]The wheat germ amylase was bound to the alginate magnetite beads as described in Materials and Methods. Elution of the bound enzyme activity was carried out by incubating the enzyme-bound beads with 1 mL of 1 *M* maltose (in the above buffer) for 4 h at 4°C. The eluate was dialyzed extensively against the above buffer and checked for enzyme activity.

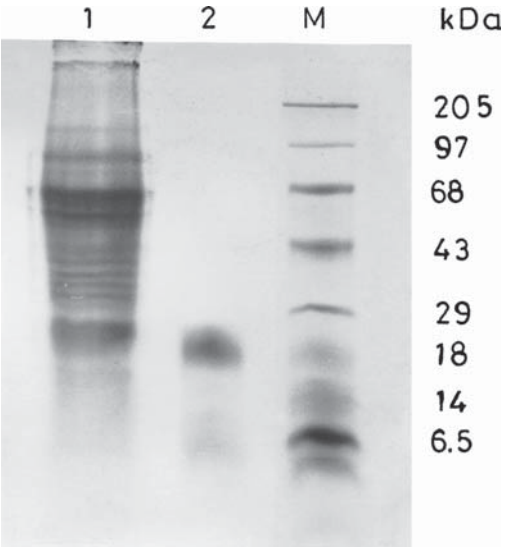

Fig. 5. SDS-PAGE pattern of wheat germ amylase. Lane 1, crude wheat germ amylase; lane 2, purified protein preparation; lane M, marker proteins.

alginate beads were used in a fluidized bed to purify wheat germ amylase, 58-fold purification with 90% activity recovery could be obtained *(16)*. The same purification has also been carried out using alginate but in an affinity precipitation format. However, an ammonium sulfate precipitation was used as an additional step before carrying out the affinity precipitation step. About 68-fold purification with 72% recovery of enzyme activity was reported *(15)*. Such data give a comparative picture of the results possible with different approaches with the same system.

Considering that for most of the food-processing applications, one does not require a homogeneous enzyme (but merely one of high activity), the single-step magnetic separation reported here seems to be a useful approach for purification of α-amylases.

Acknowledgments

Some of the results reported in this work were presented as a poster in the Proceedings of the 2nd Conference on Magnetic Separations in Biosciences and Biotechnologies. This work was supported by the Council for Scientific and Industrial Research, India. We also acknowledge the support of the organization to S.T. in the form of Senior Research Associateship.

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